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Specificity in S-Nitrosylation: A Short-Range Mechanism for NO Signaling?

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Abstract

Significance: Nitric oxide (NO) classical and less classical signaling mechanisms (through interaction with soluble guanylate cyclase and cytochrome *c* oxidase, respectively) operate through direct binding of NO to protein metal centers, and rely on diffusibility of the NO molecule. S-Nitrosylation, a covalent post-translational modification of protein cysteines, has emerged as a paradigm of nonclassical NO signaling. ***Recent Advances:*** Several nonenzymatic mechanisms for S-nitrosylation formation and destruction have been described. Enzymatic mechanisms for transnitrosylation and denitrosylation have been also studied as regulators of the modification of specific subsets of proteins. The advancement of modification-specific proteomic methodologies has allowed progress in the study of diverse S-nitrosoproteomes, raising clues and questions about the parameters for determining the protein specificity of the modification. ***Critical Issues:*** We propose that S-nitrosylation is mainly a short-range mechanism of NO signaling, exerted in a relatively limited range of action around the NO sources, and tightly related to the very controlled regulation of subcellular localization of nitric oxide synthases. We review the nonenzymatic and enzymatic mechanisms that support this concept, as well as physiological examples of mammalian systems that illustrate well the precise compartmentalization of S-nitrosylation. ***Future Directions:*** Individual and proteomic studies of protein S-nitrosylation-based signaling should take into account the subcellular localization in order to gain further insight into the functional role of this modification in (patho)physiological settings. *Antioxid. Redox Signal.* 19, 1220–1235.

Introduction

Nitric oxide (NO) is clearly recognized as a signaling molecule in different pathways. A hallmark in this recognition came from the identification of NO as the endothelial-derived relaxing factor (EDRF), a factor produced by endothelial cells that induced vascular relaxation by operating on smooth muscle cells (52, 68). It was the first mammalian gas molecule discovered as a second messenger in a signaling pathway that included its production from L-arginine by a family of nitric oxide synthases (NOSs), and generation of cyclic GMP (cGMP) after the activation of soluble guanylate cyclase (sGC) by NO binding through a high-affinity metal coordination bond (26, 127). This may be considered as the "classical" mechanism of NO signaling (103, 106), and it includes a clear example of paracrine signaling, as NO produced in endothelial cells reacts with sGC located in smooth muscle cells, taking advantage of its ability to diffuse across biological membranes. In a similar way, NO produced in one neuron diffuses and acts upon surrounding neurons. Another well-established but "less classical" signaling mechanism of NO operates through the inhibition of cytochrome *c* oxidase, the complex IV of the oxidative phosphorylation system. Both signaling mechanisms rely on direct binding of NO to protein

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metal centers through coordination chemistry (reviewed in Refs. 103–106).

In addition, several "nonclassical" nitric oxide signaling mechanisms have been described, which rely mainly on covalent post-translational protein modification by a series of reactive nitrogen species (RNS) derived from the reaction of NO with other small molecules, including free radicals. Tyrosine nitration is associated with formation of peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), and is considered mainly as an irreversible modification that can impact on some signaling pathways (69, 124, 133, 145). Cysteine residues can be oxidized following RNS formation: $ONOO^-$ induces formation of oxygenated forms (sulfenic acid, $-SOH$; sulfinic acid, $-SO_2H$; sulfonic acid, $-SO_3H$), and S-glutathionylation and other forms of S-thiolation are induced both by $ONOO^-$ and nitrosothiol formation (3, 103, 105, 106, 111, 134, 157, 163).

S-Nitrosylation (also called S-nitrosation; see Refs. 42, 73, 104 for a discussion of the terminology) has emerged as one of the main mechanisms of nonclassical NO signaling. It implies the formation of a nitrosothiol (or thionitrite, R-S-N=O) at a cysteine residue, a subtle modification that has been shown to alter the functionality of a number of proteins. There are several reviews that highlight the particular characteristics of S-nitrosylation supporting its relevance as a mechanism of redox signaling related to NO production, as well as its implications in (patho)physiology in several species (35, 44, 61, 73, 83, 90, 104, 105, 141, 160). At least for some proteins, it has been shown that S-nitrosylation leads to disulfide formation (including S-glutathionylation), so it could be considered as an intermediate to such more stable modifications (reviewed in Refs. 73, 103, 105). For each case, detailed studies could establish if the different PTMs may have the same or different functional consequences, but it seems clear that this possibility has be integrated when studying signaling by S-nitrosylation.

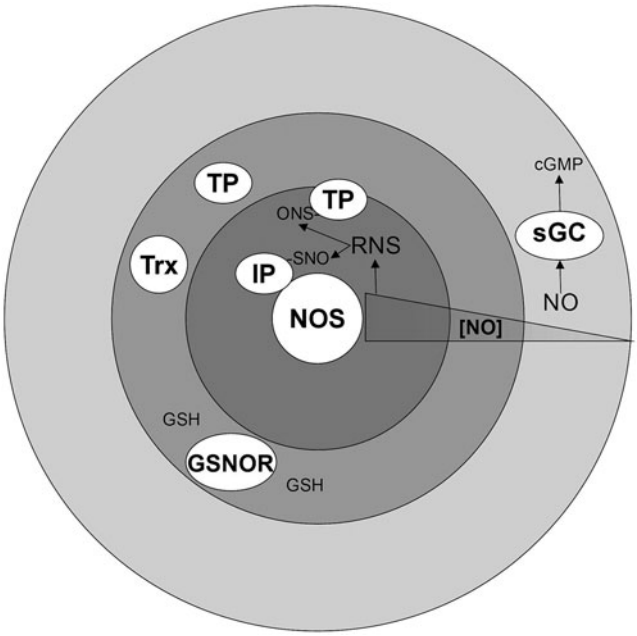

FIG. 1. Short-range and long-range NO signaling. Classical NO signaling, such as sGC activation, can be exerted at a relatively long distance from NO sources (NOS enzymes), even if NO concentration diminishes while targets are farther from the NOS. We postulate that S-nitrosylation of target proteins (TP) is essentially a short-range mechanism, limited to a tiny sphere around NOS. Among other factors described in the text, RNS formation requires higher NO concentrations, which are easier to achieve in the NOS surroundings (this is more clear in interacting proteins, IP), and denitrosylases such as Trx or GSNOR with GSH can narrow the range of action by reducing target protein S-nitrosylation.

Our aim is to review the factors that confer specificity to S-nitrosylation in order to be considered a signaling mechanism, with the proposal that its signaling function is exerted mainly in the short range, close to the NO producing sources (mainly NOS enzymes), and thus very dependent on their precise subcellular localization (Fig. 1). In addition, we provide some examples in which this short-range signaling occurs in the context of specific human and mammalian cell systems.

Biochemistry of S-Nitrosylation Specificity

Nonenzymatic biochemistry of S-nitrosylation formation

Several biochemical mechanisms have been postulated for nitrosothiol formation without requiring the presence of enzymes to catalyze it. Direct reaction of NO with cysteine residues is only relevant when the thiyl radical (P-S•) has been formed,

$$\text{P-S}\bullet + \bullet\text{NO} \rightarrow \text{P-S-NO}$$

which is relatively rare. This poses a substantial difference with classical and less classical NO signaling mechanisms, which are based on the direct reaction of NO with target proteins, and in which specificity relies on structural determinants that favor NO binding to particular metal centers. In the case of S-nitrosylation, the formation of other RNS is required, as the result of the reaction of NO with other species.

Reactions of NO with O_2 form a series of nitrogen oxides, with different oxidation states for the N atoms; among them, N_2O_3 is considered as a main nitrosating agent producing nitrite and a nitrosothiol:

$$N_2O_3 + \text{RSH} \rightarrow \text{RSNO} + H^+ + NO_2^-$$

However, the implication of N_2O_3 as a relevant nitrosating agent in biochemical environments has been challenged, mainly because its formation depends on the reaction of NO and O_2, also called NO auto-oxidation

$$2\ \text{NO} + O_2 \rightarrow 2\ NO_2$$

$$NO_2 + \text{NO} \rightarrow N_2O_3$$

and the reaction rate for its formation in water has been estimated as Rate $= k\ [\text{NO}]^2\ [O_2]$ with k being around $2–5 \times 10^6$ $M^{-2}\ s^{-1}$, consistent with the rate-limiting formation of NO_2 (11, 48, 165). Kinetic models integrating these reactions with the formation of other RNS have shown that N_2O_3 formation would not be very important at the NO concentrations estimated for biological systems (82, 87).

However, two facts can be considered that argue in favor of the relevance of this mechanism in localized cellular

environments. First, the reaction rate is very sensitive to the concentrations of the reactants, particularly NO. In a cell system where reactants concentrations are not in equilibrium, NO concentration would be much higher in a relatively small virtual sphere surrounding the NOS enzymes, allowing for faster formation of N_2O_3 and thiol S-nitrosylation. Second, it has been shown that in hydrophobic environments such as biological membranes, the same third-order rate law is observed, but the rate constant is increased up to 300 times (97, 113, 114); even with this rate acceleration, the overall NO fate would not be affected, but the reaction would be relevant enough to produce localized nitrosating species in membrane regions (114), where NOS enzymes localize due to their membrane binding structures.

An alternative mechanism for S-nitrosylation by nitrogen oxides involves the thiyl formation by NO_2 and subsequent reaction with NO (76):

$$NO_2 + RSH \rightarrow R\text{-}S\bullet + NO_2^- + H^+$$

$$NO + R\text{-}S\bullet \rightarrow RSNO$$

Again, the rate limiting step is the formation of NO_2, and it also requires an additional NO molecule, so the same considerations for the localized formation of N_2O_3 apply to this mechanism.

Metal-catalyzed formation of nitrosothiols has been described, mainly explained by a mechanism involving a one-electron oxidation of NO by oxidized transition metals, such as Fe^{3+} or Cu^{2+}; the nitrosonium (NO^+) formed could nitrosate a thiol in the proximity of such catalytic center (it is a relatively unstable species):

$$Me^{(n+1)+} + NO \rightarrow Me^n\text{-}NO^+$$

$$Me^n\text{-}NO^+ + RSH \rightarrow RSNO + Me^n + H^+$$

This mechanism has been shown to take part in specific proteins, where it can be considered as an enzymatic "nitrosylase" mechanism, such as in auto-nitrosylation of hemoglobin and other globins, and in S-nitrosoglutathione (GSNO) formation by ceruloplasmin or cytochrome *c* (reviewed in Refs. 5, 23, 53).

Recently, another pathway for S-nitrosylation formation has been described that could operate through generation of dinitrosyl-iron complexes (DNIC) from NO (20), and evidence has been found that DNIC can be a main form of NO in cells (62). This pathway will certainly deserve further investigation to assess the possible physiological role of these complexes as catalyzers of S-nitrosylation, and to test whether they are modified by subcellular localization.

Transnitrosylation

The nitroso group can be easily transferred between thiol groups, in a reversible reaction called transnitrosylation (or transnitrosation):

$$RSNO + R'SH \rightarrow RSH + R'SNO$$

Transnitrosylation among low-molecular-mass (LMM) thiols and protein thiols has been studied for a long time. LMM nitrosothiols, and especially GSNO (as the main intracellular LMM thiol is glutathione) can be considered as putative S-nitrosylation vectors in cells that could transmit the S-nitrosylation signal from the nitrosothiol-producing foci to potential protein targets. A recent study that analyzed the structural features of confirmed protein S-nitrosylation sites in the literature has postulated that a subset of proteins and residues could be specifically transnitrosylated by GSNO (101). However, the reaction of GSNO with protein thiols is more complicated, as it can also form a mixed disulfide by S-glutathionylation (reviewed in Refs. 80, 103, 105, 111), most probably with formation of nitroxyl (HNO):

$$PSH + GSNO \rightarrow PSNO + GSH$$

$$PSH + GSNO \rightarrow PSSG + HNO$$

Thus it would be interesting to compare the structural features regulating the balance between these reactions.

Several groups have recently described transnitrosylation reactions between proteins. These include protein–protein interaction determinants as specificity factors, as is the case in other enzymatic post-translational modifications used in signal transduction, such as phosphorylation. Each of the described transnitrosylases may have a restricted or broader range of target proteins and residues that can be preferentially S-nitrosylated due to their specific interactions. The nitroso group is transferred by equilibrium reactions and thus the thermodynamic comparison between both reactions (which can also be described with the redox potentials) has also to be considered. Recent reviews have thoroughly covered protein–protein transnitrosylation (5, 119), so we will just briefly describe the most relevant transnitrosylases.

Early studies on S-nitrosylated hemoglobin (SNO-Hb) reported that it was able to release NO to the blood flow causing an increase in blood pressure (50, 75, 142). Hb presents a heme group in each of its four subunits with a central iron atom, where O_2 binds. When O_2 concentration is low, NO binds to the iron; by an auto-S-nitrosylation reaction, this NO moiety is transferred to Cys93, and Hb suffers a conformational change from relaxed to tense structure. A clue for the export of the NO moiety came from the description of transnitrosylation from SNO-Hb to the most abundant protein in red blood cells, the Anion Exchanger 1 (AE1) (128). SNO-Hb interacts with AE1 in the erythrocyte membrane, promoting its transnitrosylation; subsequently the NO moiety leaves AE1 and diffuses into vessels (128). However, how this NO moiety is transferred from AE1 protein to vessels remains unclear.

Caspase-3 inactivation by S-nitrosylation of an active site Cys (Cys163) is reverted during Fas-induced signaling (100). The thioredoxin (Trx) system was described as a caspase-3 activator by denitrosylation via two different biochemical mechanisms (112). The first one implies thiol reduction in a "classical" way by means of its two active site Cys (Cys32 and Cys35) (8, 86). The second one is a transference of the NO moiety from caspase-3 to Trx by transnitrosylation of Cys32 in Trx. S-Nitrosylation of nonactive Cys has also been described, particularly Cys62, Cys69, and Cys73, in conditions where active site cysteines were oxidized (54, 59, 166). A recent study, using C32S and C35S mutants, demonstrated that Trx S-nitrosylated in Cys73 is able to transnitrosylate many cellular targets including caspase-3 (112, 166, 168). Interestingly, when Trx is S-nitrosylated in Cys73, the disulfide bond in the active site C32–C35 is not reduced by Trx reductase; both

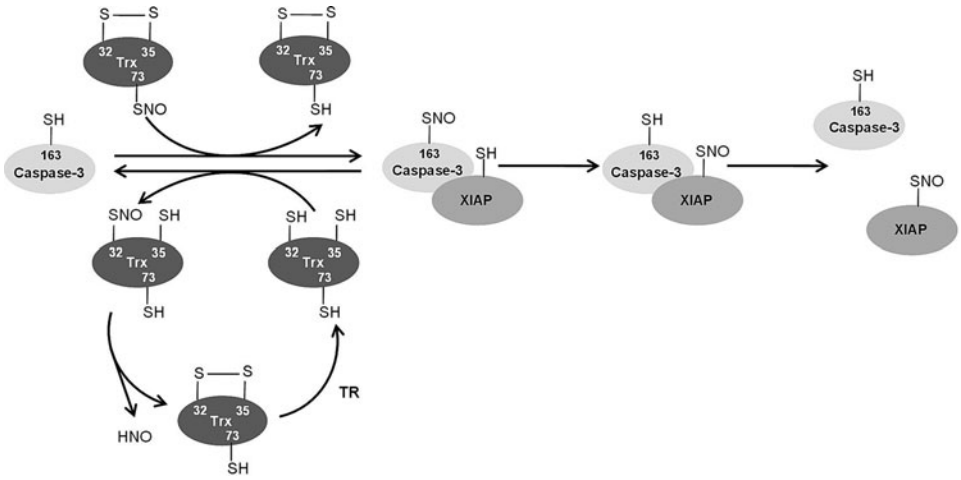

FIG. 2. Summary of transnitrosylation reactions between Trx, caspase-3, and XIAP that leads to caspase-3 activity regulation, including Cys residues that undergo S-nitrosylation. The active site of Trx denitrosylases caspase-3 in normal conditions, producing HNO and its own disulfide, which is reduced back by Trx reductase (TR). When its active site Cys is oxidized, Trx S-nitrosylated in Cys73 is able to transnitrosylate caspase-3 in Cys163. S-nitrosylated caspase-3 may also transnitrosylate its inhibitor XIAP, leading to a release of caspase-3.

enzymes are then uncoupled, which can be a mechanism to prevent the denitrosylase activity of Trx while promoting its transnitrosylase activity (59). Figure 2 summarizes the transnitrosylation and denitrosylation mechanisms involved in caspase-3 activation and inhibition in physiological and stress conditions.

Recently, S-nitrosylation of XIAP, one of the most important caspase-3/7/9 inhibitory proteins, was described in neurons (120, 155). As a consequence of this nitrosylation, the E3 ubiquitin ligase activity of XIAP is decreased and caspase degradation is inhibited, favoring apoptosis. This process is especially important in neurodegenerative diseases such as Alzheimer (AD), Parkinson (PD), and Huntington (HD) diseases, where a high content of SNO-XIAP was observed, suggesting that this reaction contributes to neuronal damage in these diseases. Interestingly, caspase-3 is able to transnitrosylate XIAP in pathophysiological situations of nitrosative stress or excitotoxicity, promoting apoptosis both by the protease activity of caspase-3 and impairing its degradation by inhibition of XIAP (120, 155).

In AD, amyloid-β peptide oligomerization provokes an increase in NO production, which leads to S-nitrosylation of Cdk5 (132). SNO-Cdk5 transnitrosylates dynamin related protein 1 (Drp1) in Cys644 (28). This reaction causes a mitochondrial fission hyperactivation as well as a compromise in mitochondrial bioenergetics which, in synaptic structures, could lead to synaptic loss (28, 159).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also implicated in transnitrosylation processes. GAPDH is S-nitrosylated in its Cys150 residue; S-nitrosylated GAPDH interacts with Siah1, and it is translocated to the nucleus due to Siah1 nuclear localization signal (56). Once in the nucleus, SNO-GAPDH transnitrosylates proteins such as SIRT1, HDAC2, and DNA-PK, affecting metabolic pathways, normal aging processes, chromatin remodeling in neuronal development, or neurodegeneration (81, 121).

Transnitrosylation has been involved in the formation of pulmonary arterial hypertension (PAH). Mice chronically treated with N-acetylcysteine (NAC) or its S-nitrosylated form, S-nitroso-N-acetylcysteine (SNOAC), developed PAH, mimicking the effects of chronic hypoxia (126). This study showed that NAC needs to be converted to SNOAC and that activation of the HIF-dependent hypoxia response through transnitrosylation of particular proteins in the canonical HIF activation pathway (such as pVHL) could be among the molecular mechanisms responsible for this effect (126).

Denitrosylases

Removal of the nitroso group is another important aspect of S-nitrosylation signaling. It is generally accepted that S-nitrosylation is a labile modification, and that the levels of cellular nitrosothiols are low, due to a rapid turnover. Denitrosylation was firstly conceived as an unregulated and spontaneous process, and several nonenzymatic mechanisms of denitrosylation have been described that could potentially act *in vivo*. These include reactions mediated by nucleophilic compounds, transition metal ions, reactive oxygen species (ROS), and ascorbate (reviewed in Ref. 147).

Accumulating evidence shows that several enzymes can catalyze denitrosylation *in vitro* (14, 64, 77, 143, 154) and *in vivo* (16, 95) (reviewed in Refs. 5, 17). These enzymes may contribute to spacially limit the action of S-nitrosylation events, helping to keep their precise subcellular localization close to NO sources. They also may help to protect cells from ''excessive'' S-nitrosylation, as, for example, when inducible nitric oxide synthase (iNOS) is induced in immune cells in order to attack pathogens (reviewed in Ref. 60). In addition, they provide another mechanism of specificity and regulation since these enzymes may act over defined sets of substrate proteins. This is more important in examples of tightly regulated denitrosylation that may activate precise signals, such as the case of Fas-induced caspase-3 denitrosylation (100), which can be mediated by Trx (16), or receptor-regulated endothelial nitric oxide synthase (eNOS) activation (37).

Two main enzymatic mechanisms of denitrosylation have emerged in recent studies, GSNO reductase and the thioredoxin system. GSNO reductase (GSNOR) is an evolutionary conserved enzyme system previously known as alcohol dehydrogenase class III. GSH can react with a protein nitrosothiol leading to either transnitrosylation and subsequent generation of S-nitrosoglutathione (GSNO) or glutathionylation of the protein, in both cases with subsequent

release of the NO group from the protein thiol. This is supported by the observation that addition of GSH to SNO-proteins leads to their denitrosylation (125). GSNOR mainly catalyzes the denitrosylation of GSNO towards GSSG, utilizing NADH as an electron donor. The cycle is completed as GSSG is reduced by glutathione reductase and NADPH. Although GSNOR does not directly act on protein nitrosothiols, an equilibrium between GSNO and SNO-proteins is maintained by transnitrosylation, since *GSNOR*-knockout mice showed increased levels of both GSNO and SNO-proteins after nitrosothiol addition or lipopolysaccharide (LPS) treatment (95, 96).

GSNOR participates in signal transduction through G protein-coupled receptors (GPCRs) and, in particular, β_2-adrenergic receptor (β_2-AR). Upon agonist stimulation, GPCR kinases (GRKs) 2 and 3 are recruited to the plasma membrane by interaction with G$\beta\gamma$ subunits and phosphorylate the β_2-AR at the cytoplasmic tail (Fig. 3). This allows β-arrestin 2 to be transiently recruited by the activated receptor and driven to clathrin-coated pits where, after its dissociation, the receptor internalizes and becomes desensitized (reviewed in Ref. 135). Both GRK2 and β-arrestin 2 S-nitrosylations are controlled by GSNOR since both augment in *GSNOR*-knockout mice. S-Nitrosylation at GRK2 Cys340 decreased its kinase activity, β-arrestin 2 interaction and β_2-AR internalization and desensitization (164). On the other hand, binding of β-arrestin 2 to the clathrin pit is promoted by S-nitrosylation, which in turn increases β_2-AR internalization (123). This differential regulation of GPCR signaling by S-nitrosylation suggests that local denitrosylation of particular elements becomes important for cell signaling. GSNOR has been linked with the development and protection against several diseases. S-Nitrosylation of O6-alkylguanine-DNA alkyl transferase (AGT) in the liver of $GSNOR^{-/-}$ mice after LPS or diethylnitrosamine treatment promoted decreased capability of DNA repair, thus increasing hepatocellular carcinoma incidence (162). On the other hand, GSNOR deficiency protected against myocardial infarction (89).

The other best known denitrosylase is the thioredoxin (Trx) system. While GSNOR denitrosylates SNO-proteins indirectly through GSNO formation, Trx system has been shown to denitrosylate them directly. As mentioned above, uncoupled Trx can act as a transnitrosylase when its Cys73 is S-nitrosylated, after disulfide formation between catalytic Cys32 and Cys35; however, for its denitrosylase function it uses the catalytic center with both Cys32 and Cys35 in their reduced state (167). Trx establishes a disulfide bridge with the substrate SNO-protein allowing the release of the NO (in the form of HNO) (16). An intramolecular disulfide bond is then formed between Cys32 and Cys35, substituting the intermolecular disulfide. Thioredoxin reductase (TR) is in charge of restoring the reduced state of these Cys through a NADPH–dependent reduction of the Trx intramolecular disulfide.

Trx1 was identified as the caspase-3 constitutive and cytosolic denitrosylase since inhibition of Trx1 or TR1 produced increased amounts of SNO-caspase-3 (16). On the other hand, Trx2 was identified as a denitrosylase of mitochondrial caspase-3 upon Fas ligand stimulation, a process required for its activation and, therefore, for apoptotic cell death (16). Furthermore, the Trx-interacting protein (Txnip) has been shown to inhibit denitrosylate activity of Trx, since it increases SNO-protein levels (43).

Proteomic analyses have helped to highlight Trx as a denitrosylase. In cytokine-activated macrophages where iNOS is induced, we used a "fluorescence switch" technique to label S-nitrosylated proteins, identifying eleven proteins that were differentially S-nitrosylated when the thioredoxin reductase was inhibited with auranofin (152). Benhar *et al.* used a quantitative LC-MS/MS approach to identify proteins that were denitrosylated when they added Trx/TR to cell extracts that had been nitrosylated *in vitro* (18). Interestingly, Trx targets of transnitrosylase activity could be differentiated from those of denitrosylation, providing specificity to the search of Trx substrates in the context of S-nitrosylation (168).

Denitrosylation has emerged as a relevant mechanism by which functional relevance of S-nitrosylation is regulated.

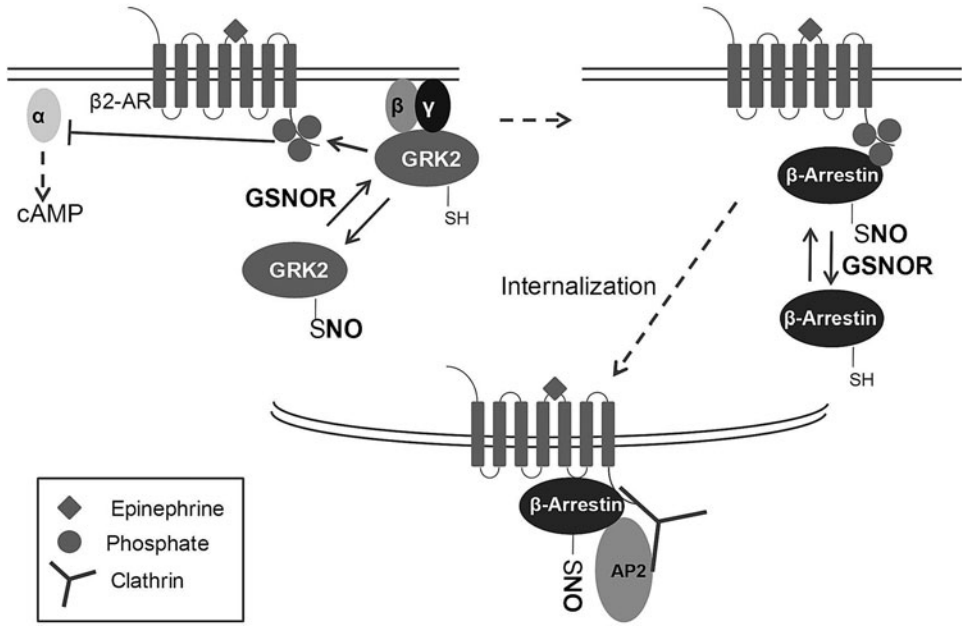

FIG. 3. Agonist-stimulated internalization of β_2-AR is regulated by S-nitrosylation. After agonist (epinephrine) stimulation, β_2-AR stimulates separation of α from β and γ subunits of the G protein. The two latter subunits bind reduced GRK2, which phosphorylates β_2-AR. S-Nitrosylation of GRK2 prevents its binding to β and γ subunits. Once phosphorylated, β_2-AR recruits β-arrestin which becomes S-nitrosylated and subsequently binds to AP2 and clathrin, thus allowing internalization. If β-arrestin is not S-nitrosylated, internalization of β_2-AR becomes attenuated.

Study of the dynamic equilibrium between nitrosothiol generation and its removal, as well as the enzymes implicated will provide more accurate knowledge of S-nitrosylation regulation *in vivo* and their connection with diseases.

Regulation of S-Nitrosylation by Subcellular Localization of eNOS

Despite some evidence indicating a possible role for the compartmentalization of iNOS and short-range actions of high levels of NO in protein S-nitrosylation—for instance, in the S-nitrosylation of matrix metalloproteinase 9 (MMP-9) at the leading edge of migrating cells, and chloride intracellular channel 4 (CLIC4) during its nuclear translocation in macrophages (58, 99)—eNOS and neuronal nitric oxide synthase (nNOS) are the prototypic NOS whose dependence between localization and S-nitrosylation has been more extensively studied (70, 151).

In cells, eNOS traffics between the plasma membrane and the cytoplasmic face of the Golgi apparatus by means of both specific interaction with proteins involved in vesicle trafficking and a cycle of palmitoylation/depalmitoylation on Cys15/Cys26, two amino acid residues whose mutations do not affect the catalytic activity of the enzyme itself but decrease NO production in cells due to alteration of eNOS localization (36, 94, 122, 146). A large body of work indicates that the confinement of eNOS in cell compartments and its proximity or binding to target proteins may lead to selective S-nitrosylation.

eNOS at the plasma membrane

Several studies have shown that preferential localization of eNOS at the plasma membrane produces higher levels of NO and S-nitrosylation in this region than in the Golgi apparatus. At the plasma membrane, eNOS is retained in its low active state associated to caveolin-1 in caveolae, and it is fully activated after dissociation from caveolin-1 by Ca^{2+}/calmodulin binding and kinase-mediated phosphorylation on key serine residues (Ser1177, Ser635, and Ser617); among them, phosphorylation of Ser1177 by Akt is particularly remarkable as the most constant sign of eNOS activation (36, 46). In contrast, the association of eNOS with the actin cytoskeleton by means of its interaction with NOS-interacting protein (NOSIP) or its trafficking towards the Golgi complex by binding to NOS traffic inducer (NOSTRIN) can reduce eNOS activity, moving the enzyme away from plasma membrane-associated signals of activation (122). eNOS activity can also be negatively regulated through auto-S-nitrosylation on Cys94 and Cys99, a negative feedback mechanism that affects NO synthesis by impairing substrate binding and/or electron transfer at the eNOS dimeric interface (36). Studies carried out with both plasma membrane-restricted eNOS and a cytoplasmic mutant deficient in myristoylation on Gly2—which alters its membrane binding and inhibits further palmitoylation—clearly show that membrane localization is required for S-nitrosylation (37, 38). Since eNOS can be reversibly S-nitrosylated, compartmentalized S-nitrosylation of eNOS might be favored, for instance, by environments devoid of denitrosylases such as thioredoxin or GSNOR. However, complementary studies carried out with compartment-targeted iNOS constructs and calcium-independent eNOS showed no differences between the activities of these mutants irrespectively of their subcellular localization (32, 72, 131), suggesting that the levels of NO produced are the most important factors for localized S-nitrosylation and that, in the case of constitutive/regulatable NOS, proximity to the upstream signaling pathways—Ca^{2+}/calmodulin and Ser/Thr kinases—may explain the differences in NO production and S-nitrosylation observed between membrane cell compartments.

In addition to auto-S-nitrosylation of eNOS, other proteins involved in what can be called the "eNOS system" are targets of S-nitrosylation, which can alter the system activity. The eNOS-binding protein caveolin-1 is constitutively S-nitrosylated in endothelial cells (117), although if this can regulate eNOS localization and activity has not been described, to our knowledge. The chaperone Hsp90 also interacts with eNOS, increasing its affinity for calmodulin and Akt, and thus its activity (51, 149). We have shown that Hsp90 can be S-nitrosylated in Cys597 (107), an amino acid residue located in the region of interaction with eNOS (40). This modification inhibits the ATPase activity of Hsp90, and its ability to activate eNOS, representing an additional S-nitrosylation-based feedback mechanism modulating eNOS activity. It is worth noting that the binding of Hsp90 to NOS is not an exclusive hallmark of eNOS since both nNOS and iNOS can also be activated by binding to Hsp90 (129, 169). Whether nNOS and/or iNOS may also S-nitrosylate Hsp90 in the context of a negative regulatory feedback loop of NOS activation, and whether their subcellular localization is actually important for Hsp90 S-nitrosylation deserves further research.

An increasing number of proteins S-nitrosylated by eNOS at the plasma membrane have been identified over the last years. eNOS can interact with the GTPase dynamin-2, increasing receptor-mediated endocytosis through S-nitrosylation of Cys607, a modification that fosters dynamin oligomerization and GTPase-dependent vesicle scission from the plasma membrane (25, 158). Interestingly, a recent report suggested a role for eNOS and dynamin-2 in the immune-escape of uropathogenic strains of *Escherichia coli* (161). The study shows that *E. coli* invasion of bladder epithelial cells in recurrent urinary tract infections is facilitated by endocytosis mediated by eNOS-dependent S-nitrosylation of dynamin-2. It would be important to address if this is a general mechanism of escape for those microorganisms infecting hosts through NO-producing specialized barriers. β-Arrestin is also regulated by eNOS-dependent S-nitrosylation; it forms a complex with eNOS that translocates to GPCRs upon agonist binding, resulting in eNOS activation and β-arrestin S-nitrosylation (123). Once β-arrestin is S-nitrosylated, eNOS dissociates from the complex, and stimulated GPCRs can internalize by association with the clathrin-β-arrestin endocytic machinery (123). β-Catenin, an essential component of adherens junctions—intercellular structures controlling permeability on epithelial and endothelial monolayers—is also a target of S-nitrosylation by eNOS. Stimulation of endothelial cells with VEGF induces eNOS-dependent S-nitrosylation of β-catenin on Cys619, an important amino acid residue for the interaction with VE-cadherin and the preservation of the permeability cell barrier (153). Up to our knowledge, it is not clear yet if there is a direct interaction between eNOS and β-catenin. H-Ras, a Ras isoform localized both at the plasma membrane and the Golgi complex, has recently been added to the list of proteins that can be activated at the plasma membrane by eNOS-dependent S-nitrosylation. In endothelial cells, bradykinin stimulation

induces preferential activation of H-Ras at the plasma membrane through a mechanism independent of Src activation which involves S-nitrosylation of H-Ras on Cys118 (15).

N-Ethylmaleimide-sensitive fusion protein (NSF), a SNARE regulator involved in vesicular trafficking and exocytosis of platelet α-granules and Weibel-Palade bodies from endothelial cells, is also S-nitrosylated by eNOS as demonstrated using NOS inhibitors and eNOS-deficient cells (109, 116). S-Nitrosylation of NSF reduces granule exocytosis by stabilizing SNARE complexes in vesicles. The possible role played by the compartmentalized activity of eNOS on NSF S-nitrosylation has been studied, trying to discriminate the effects of Golgi and plasma membrane localization. Whereas a recent study with eNOS directed to either plasma membrane or Golgi found that eNOS induces higher levels of NSF S-nitrosylation at the plasma membrane (131), imaging analysis from previous studies carried out with wild-type eNOS-transfected COS-7 cells clearly showed that in response to ATP eNOS produced NO mainly on the Golgi complex, a compartment in which most of eNOS and S-nitrosylated proteins are concentrated (71). In this regard, NSF trafficking may play an important role in a cellular model of pulmonary arterial hypertension induced with the pirrolizidine alkaloid MCTP, which induces megalocytosis of pulmonary arterial endothelial cells (117, 118). These studies show that MCTP disrupts vesicular trafficking in endothelial cells, mislocalizing eNOS in the cytoplasm accompanied by loss of NO from caveolae and reduced S-nitrosylation and localization of NSF on the Golgi. Whether reduced NSF S-nitrosylation in MCTP-induced aberrant trafficking is the consequence of eNOS dysfunction at the plasma membrane, the Golgi complex, or both remains an open question.

eNOS on the Golgi

Several studies have also shown that eNOS localization on the Golgi complex is functional and contributes to S-nitrosylation of Golgi-localized proteins (70). Although the mechanisms involved in eNOS localization on the Golgi complex are not fully understood, it is clear that palmitoylation on Cys15/Cys26 and NOSTRIN binding to eNOS play an important role. In this regard, a recent study shows that activation of the transcription factor STAT-3 by the adhesion molecule PCAM-1 induces NOSTRIN expression and the traffic of eNOS from the plasma membrane towards the Golgi complex (110). The role of subcellular localization on eNOS activation has been studied by transfecting protein versions with different localization signals. Transfection in COS-7 cells showed that cis-Golgi-targeted eNOS was less sensitive to activation by Ca^{2+}/calmodulin but more easily activated by Akt-mediated phosphorylation on Ser1179, when compared to the protein localized on the plasma membrane (45). Similar cell transfection studies performed in eNOS-deficient endothelial cells showed that on the Golgi complex eNOS may also result less sensitive to Akt activation but resistant to cholesterol and LDL inhibition, releasing lower amounts of NO than at the plasma membrane (170). Altogether, these reports suggest that compartmentalized activity of eNOS on the Golgi complex may be cell-type specific and/or that low to moderate levels of NO would be enough to S-nitrosylate Golgi resident proteins near eNOS, either due to an enhanced selectivity for S-nitrosylation, or to decreased denitrosylation.

A recent proteomic study combining biotin-switch assay and mass spectrometry has identified nine Golgi-resident proteins S-nitrosylated in Golgi membranes isolated from rat livers (136). Among those, the authors focused on the study of extracellular matrix metalloproteinase inducer (EMPRIN)—a member of the immunoglobulin superfamily involved in invasion and metastasis—and Golgi phosphoprotein 3 (GOLPH3), a potential oncogene involved in protein glycosylation and mTOR regulation. Both proteins interacted with eNOS and co-localized with it on the Golgi of endothelial cells, increasing their S-nitrosylation upon activation with the Ca^{2+} ionophore ionomycin and also in pathophysiological conditions such as cirrhosis (136).

In T cells, S-nitrosylation is mainly compartmentalized near the Golgi complex, where active eNOS localizes. In this compartment, eNOS S-nitrosylates N-Ras at Cys118, fostering the conversion of GDP- to GTP-bound N-Ras, and the consequent activation of the MAPKs ERK-1 and ERK-2 (67). Interestingly, although T cells express both K-Ras and N-Ras (which share the same conserved Cys residue sensitive to S-nitrosylation), only N-Ras becomes S-nitrosylated due to its preferential localization on the Golgi, suggesting that proximity of eNOS-derived NO to N-Ras is an important determinant of S-nitrosylation and MAPK activation (Fig. 4). MAPK phosphatase 7 (MKP7)—a negative regulator of the MAPK JNK3—is S-nitrosylated by eNOS on Cys244 in response to SDF-1, a CXC chemokine with proangiogenic properties on endothelial cells (130). S-Nitrosylation reduces the phosphatase activity of MKP7, increasing JNK3 activity, and thus endothelial cell migration. Although there is no evidence of compartmentalized S-nitrosylation of MKP7, since the Golgi complex plays an important role in the regulation of MAPK pathways (19), it would be interesting to explore whether eNOS-mediated MKP7 S-nitrosylation may take place on the Golgi.

Nitric Oxide Synthases and the Heart: Role of Localized S-Nitrosylation

Very early since the characterization of eNOS in endothelial cells, it was clear that this isoform was expressed in cardiomyocytes and that NO could play a role as a regulator of cardiac rhythm and inotropic responses (108, 139). It is now accepted that the heart expresses both eNOS and nNOS, the former in endothelial and endocardial cells, the latter in nervous tissue and autonomic ganglia, and both in cardiomyocytes. In addition, iNOS may be expressed upon exposure of endothelial cells or cardiomyocytes to cytokines or proinflammatory stimuli (9, 12). A significant discovery which shifted the view of the role of NO in the heart was related to the topological confinement of NO signaling, whereby eNOS, localized in caveolae, mainly directed its action towards the inhibition of β-adrenergic-induced inotropy. In contrast, nNOS, which is targeted to sarcoplasmic reticulum, stimulates calcium release via the ryanodine receptor (RyR) with an opposite effect on contractility (13). Both classical and nonclassical modes of signaling (103) have been demonstrated for NO within the heart. The classical cGMP-mediated pathway is responsible for several important actions of NO related to regulation of contractility and inhibition of cardiac remodeling (see Ref. 55 for review). Elevation of cGMP levels encompasses the activation of cGMP-dependent protein

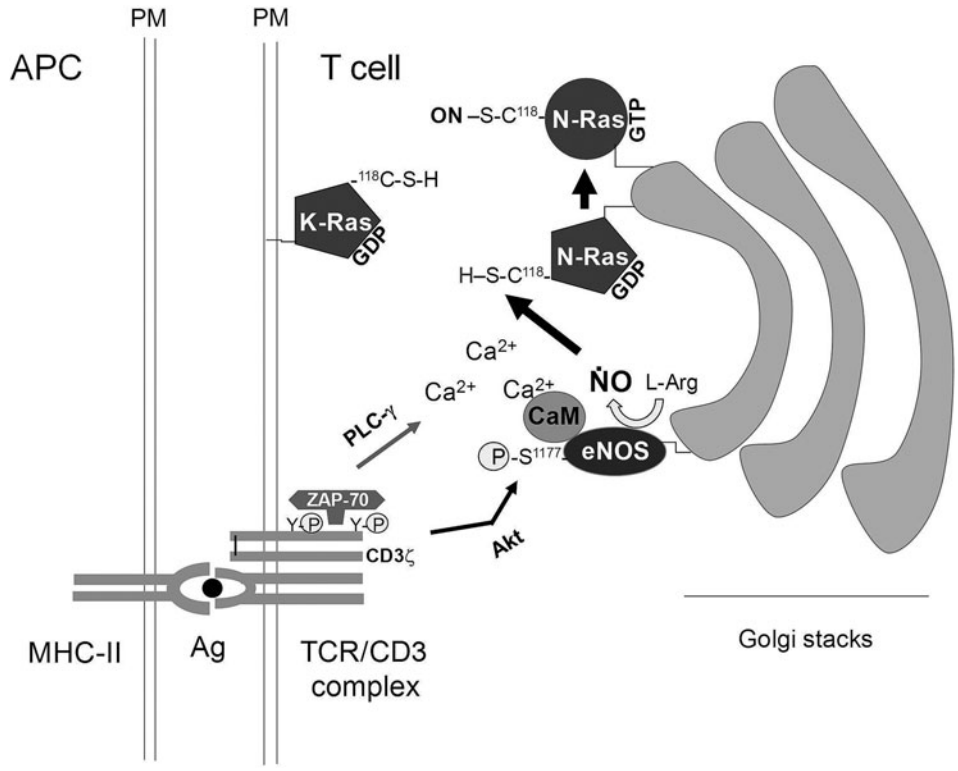

FIG. 4. Compartmentalized S-nitrosylation of N-Ras but not K-Ras in antigen-stimulated T cells. The figure represents the signaling pathways involved in the selective eNOS-dependent S-nitrosylation and activation of N-Ras on the Golgi complex. Although K- and N-Ras are both farnesylated, K-Ras is targeted to the plasma membrane (PM) by means of a basic carboxyl-terminal region of amino acids, whereas N-Ras is mainly localized on the Golgi by palmitoylation, co-localizing with eNOS. Upon TCR binding to antigen (Ag) on an antigen presenting cell (APC), the TCR complex is phosphorylated on the CD3ξ chains, which induces the activation of PLC-γ and Akt by recruitment of the tyrosine kinase ZAP-70. PLC-γ increases the cytosolic levels of inositol 1,4,5-triphosphate, releasing Ca^{2+} from internal stores, which in turn can bind calmodulin-associated eNOS. On the other hand, Akt can phosphorylate eNOS on Ser1177. As a result of the combined actions of Ca^{2+} and phosphorylation, eNOS is activated producing NO on the Golgi, fostering N-Ras activation by S-nitrosylation on Cys118, an amino acid residue shared by K-Ras but which is not S-nitrosylated in T cells due to K-Ras localization at a different cell compartment.

kinases (PKGs) that are important for the regulation of contractility through the inhibition of the L-type Ca^{2+} channel (LTCC). Noteworthy, cGMP signaling in cardiomyocytes is also compartmentalized due to specific subcellular localization of soluble and particulate guanylate cyclases and of phosphodiesterase enzymes (PDEs), thus offering another example of subcellular localization as a major means to control intracellular signaling. The nonclassical pathway is inherently related to NO-induced post-translational modifications of thiols, such as S-nitrosylation and S-glutathionylation (103, 105, 106). For a comprehensive update on the role of these PTMs in the cardiovascular system, the readers may wish to consult recent excellent reviews (102, 138).

eNOS has been shown to localize mainly within the caveolae of sarcolemma and T-tubules, which supports the idea that NO generated through this isoform exerts its action on vicinal cell surface receptors that regulate contractility, including muscarinic, β-adrenergic, and bradykinin receptors (57). The global eNOS effect is negative on chronotropy and inotropy and is counteracted by the action of nNOS, circumscribed to the sarcoplasmic reticulum. Interactions of eNOS and nNOS with other proteins sharing their subcellular localization, caveolin 3 and the RyR, respectively, are essential to understanding their action on cardiac function (156). S-Nitrosylation has been described to involve several proteins regulating contractility including LTCC, RyR, Kv1.5 channel, and sarcoplasmic/endoplasmic reticulum Ca^{2+}-activated ATPase (SERCA). The ryanodine receptor Ca^{2+} release channel (RyR2), together with SERCA2a, are critical components of the excitation–contraction coupling molecular machinery. It has been proposed that the close apposition of nNOS and RyR2 facilitates S-nitrosylation of the latter, increasing its channel opening probability (88). A similar mechanism may operate for the S-nitrosylation-induced regulation of LTCC and SERCA2a which has also been shown to become activated by NO-induced S-glutathionylation (3). A deficit of nNOS-mediated S-nitrosylation of RyR2 has been associated with pathological cardiac responses such as arrhythmia (49). Other ion channels have also been proposed to undergo S-nitrosylation: intermediate conductance potassium channel (IK1), late inward Na+ current (InaL), slowly activating delayed rectifier current (Iks), and ATP-sensitive K+ channel (see Ref. 150 for review). Finally, eNOS and vicinal scaffolding proteins are themselves potential targets for S-nitrosylation, at least in endothelial cells (37, 107), resulting in an autoinhibitory feedback mechanism. Thus, knowledge of

S-nitrosylation modification of cardiac function depicts a complex scenario which still awaits further clarification through the employment of animal models and corroboration by clinical settings where this PTM is specifically addressed.

Short-Range S-Nitrosylation Signaling in the Brain

In neurons, NO is produced essentially by neuronal NO synthase (nNOS), which can be localized in well-defined neuronal localizations, such as the presynaptic terminal or the postsynaptic density, where NO production can be coupled to synaptic transmission, either in an anterograde or retrograde manner. The best characterized signaling molecule coupled to NO production by nNOS in the brain is the neurotransmitter glutamate. Glutamate is the main excitatory neurotransmitter in the central nervous system, and is recognized by both ionotropic and metabotropic receptors. The calcium-permeable ionotropic N-methyl-D-aspartate (NMDA) receptor was shown to be associated to glutamate-induced calcium-dependent NO production by nNOS in the brain (21, 47). NMDA receptors are also central to neurodevelopment, synaptic plasticity, and neurodegeneration. Other ionotropic glutamate receptors have also been involved in these processes, such as AMPA receptors. Overactivation of glutamate receptors often results in excitotoxicity, a phenomenon caused by excitatory neurotransmitters that cause a rise in the intracellular calcium concentration induced by receptor activation, which may trigger cell death pathways (2, 41, 84).

NMDA receptor coupling to nNOS by PSD-95: Implications for neuronal survival

The participation of NMDA receptors in excitotoxic neuronal death has been extensively studied in the last 30 years, and NO has been shown to be a participant in this complex cascade of events. Glutamate release by synapses causes an increase in the intracellular calcium concentration of the postsynaptic neurons, and this rise in calcium is usually mediated by influx through voltage-dependent calcium channels, influx by calcium-permeable receptors (such as NMDA or certain types of AMPA receptors) or by reversal of the sodium–calcium exchanger (10). Activation of NMDA receptors by glutamate causes an influx of calcium through the receptor itself that was shown to be linked to the activation of nNOS and neuronal death (22). Activation of AMPA receptors was also linked to NO production by nNOS, resulting in neurotoxicity (6). NO produced by nNOS following activation of NMDA receptors was initially shown to mediate the neurotoxicity of glutamate in cultured neurons (34), and a number of studies later showed that NO produced by nNOS during a variety of brain insults is responsible, at least partially, for the neuronal damage (for review, see Refs. 2, 7, 24, 27, 78, 84).

NO is produced rapidly following stimulation of NMDA receptor, which prompted the investigation of whether a structural proximity between this glutamate receptor and nNOS existed. The PDZ domains in PSD-95 mediate a structural interaction between the NMDA receptor NR2 subunits and nNOS at the postsynaptic density (31), an area that is rich in anchoring and scaffolding proteins that help in making synaptic transmission more efficient (Fig. 5). Another study showed that uncoupling the NMDA receptor from nNOS by interfering with PSD-95 resulted in loss of the neurotoxic effect of NO (137), without loss of calcium influx through the receptor. Peptide inhibitors such as Tat-NR2B9c that disrupt the coupling of the NR2 subunits of the NMDA receptor and PSD-95 provoke a loss of NO production following NMDA receptor activation (1). Such peptide inhibitors contain a sequence similar to the C-terminal of NR2 subunits, which competes with native NMDA receptors and disrupts the interaction of NMDA receptors and PSD-95.

PSD-95 itself presents sites of competing regulation which control the targeting of PSD-95 to the postsynaptic density, on Cys3 and Cys5, which can be both regulated by S-palmitoylation (increases the localization of PSD-95 at the postsynaptic density) or by S-nitrosylation (reduces the clustering of PSD-95 on synapses), supporting a model for regulation of NMDA receptor activation coupled to NO

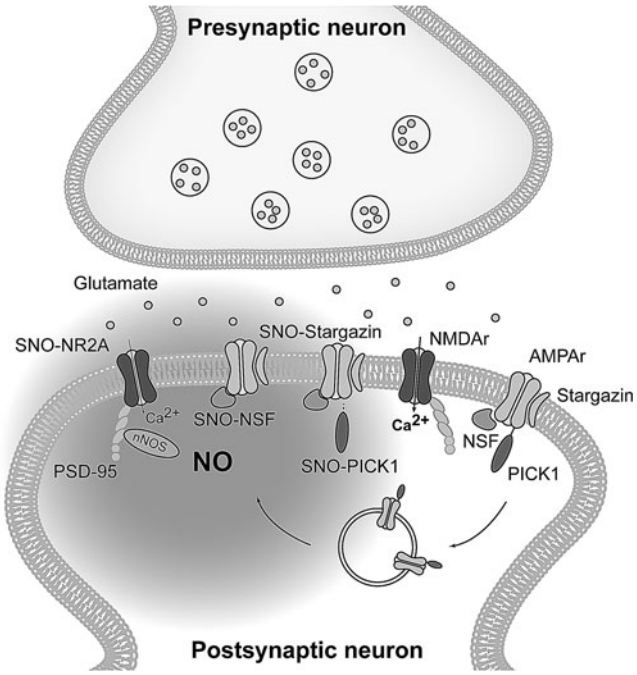

FIG. 5. Postsynaptic localization of nNOS and S-nitrosylation signaling in a glutamatergic synapse. Production of NO by nNOS following glutamate release is coupled to activation of Ca^{2+}-permeable NMDA receptors (NMDAr), which are anchored at the postsynaptic density by scaffolding proteins, including PSD-95. PSD-95 binds to NMDAr and nNOS via PDZ domains, allowing for a close proximity of permeable NMDAr and nNOS. NO produced in close proximity to NMDA receptors triggers the S-nitrosylation of NR2A subunits (SNO-NR2A), which then allow less Ca^{2+} in. Activation of NMDAr also triggers the recruitment of more AMPA receptors (AMPAr) towards the membrane surface. S-Nitrosylation of stargazin (SNO-stargazin) and NSF (SNO-NSF) contributes to increase the surface expression of AMPAr during events of synaptic plasticity following the activation of NMDAr. On the other hand, S-nitrosylation of PICK1 facilitates its release from AMPAr after membrane insertion and also facilitates surface expression of AMPAr. When these proteins are out of range of NO and are not S-nitrosylated, PICK1 interacts more strongly with the receptor while NSF interacts more weakly, allowing the binding of proteins that facilitate endocytosis of AMPAr.

production via PSD-95, by controlling the postsynaptic localization of PSD-95 as well (63).

The close proximity of nNOS and the NMDA receptor via PSD-95 in the postsynaptic density facilitates the regulatory effect of NO also on the NMDA receptor itself (29). The first reports that NO would affect redox modulatory sites of the NMDA receptor date back to the 1990s (85, 91, 93). It was then hypothesized that S-nitrosylation would decrease the permeability of the receptor, as a neuroprotective feedback mechanism (92) (Fig. 5). Site-directed mutagenesis studies later identified Cys399 in the NR2A subunit of the NMDA receptor that may undergo endogenous S-nitrosylation and thus regulate the ion channel activity, decreasing calcium influx (30, 79). S-Nitrosylation of NR1 subunits may also occur on Cys744/Cys798 pair, particularly during hypoxia or stroke conditions (148), which supports a function for S-nitrosylation of the NMDA receptor as a way to regulate calcium influx through the receptor and limit neurotoxicity.

Interestingly, it has been known for a long time that nNOS-positive neurons are relatively resistant to excitotoxic stimuli and are spared in several neurodegenerative conditions, such as Alzheimer disease (66) and Huntington disease (HD) (39). The selective resistance of nNOS-positive striatal interneurons in HD was correlated with the fact that such neurons probably have endogenous S-nitrosylation of their NMDA receptors, thus becoming less permeable to calcium influx and therefore less likely to degenerate (171). On the other hand, unlike nNOS-positive interneurons, striatal medium spiny neurons do not produce NO and are sensitive to excitotoxic damage and degenerate more easily. This may render the NMDA receptor in medium spiny neurons more permeable to calcium, and thus increase their susceptibility to injury. Furthermore, in a model of HD, it was observed that PSD-95 and nNOS are less present in association with the synaptic membrane and the NMDA receptor in striatal neurons (74), which suggests a decreased coupling between the receptor and NO production.

S-Nitrosylation and synaptic plasticity

Synaptic plasticity underlies the formation of new memories and learning processes. One form of synaptic plasticity is the insertion of synaptic AMPA receptors in postsynaptic sites following activation of NMDA receptors. The increase in AMPA receptors at the synapse makes the synapse respond with larger excitatory currents upon synaptic stimulation, thus making the synapse stronger. Several intracellular proteins are involved in the recruitment of AMPA receptors to the synaptic membrane, and some may be functionally S-nitrosylated (4, 115, 151).

Upon glutamatergic stimulation, AMPA receptors mediate the initial membrane depolarization that allows activation of NMDA receptors. Activation of NMDA receptors triggers a set of events that result in long-term changes in the number of AMPA receptors present in the synaptic membrane (98). Following activation of NMDA receptors, a number of proteins are responsible for regulating the insertion or removal of AMPA receptors to the membrane. NSF and Protein Interacting with C Kinase (PICK1) are two regulatory proteins that interact with AMPA receptors and that are sensitive to S-nitrosylation (Fig. 5). NSF is important for membrane fusion during exocytosis and was shown to be S-nitrosylated (65). In this study, the authors show that S-nitrosylation of Cys91 increases the association of NSF with GluR2-containing AMPA receptors, thus driving surface expression of AMPA receptors (65). Nitrosylation of NSF was later shown to allow unclustering of PICK1 and delivery of AMPA receptors to the membrane (144). This mechanism may be responsible for long-term potentiation dependent on activation of the NMDA receptor. Stargazin is another protein important for regulation of AMPA receptor surface expression, and physiological S-nitrosylation of stargazin at Cys302 in the C-terminal tail elicited by NMDA receptor activation was shown to increase the presence of AMPA receptors at the membrane surface (140) (Fig. 5). Interestingly, the uncoupling of NMDA-PSD-95 interaction with Tat-NR2B9c was shown to affect plasticity related to nociceptive signaling (33), but a similar approach was not yet described for synaptic plasticity. To date, of the many proteins involved in regulating AMPA receptor surface expression, only NSF, PICK1, and stargazin were shown to be functionally modified by S-nitrosylation following NMDA receptor activation and postsynaptic NO production by nNOS, but possibly other proteins may also be controlled by S-nitrosylation with implications for synaptic plasticity.

Conclusions

Protein S-nitrosylation is generally accepted as a nonclassical mechanism of NO signaling. Although the precise biochemical pathways leading to its formation are not completely understood, several of the pathways that have been so far described point out to the hypothesis that this modification is mainly localized close to the NO producing foci, which in

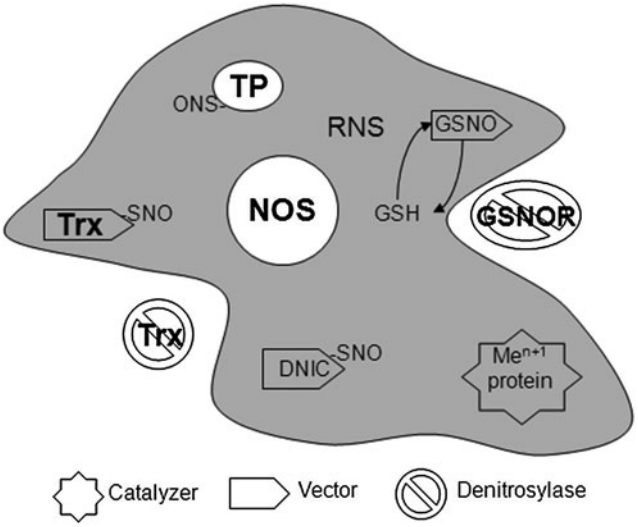

FIG. 6. Integration of several factors affecting short-range S-nitrosylation signaling. The short-range S-nitrosylation signaling range of action shadowed in gray (see also Fig. 1) becomes more complex when other factors are included. In addition to catalyzed RNS formation that could be exerted by membranes (not shown), some metal proteins are also catalyzers of S-nitrosylation formation, expanding the range of action. Transnitrosylases (including Trx) and other LMM vectors such as GSNO or DNIC can also selectively extend it to their targets. On the other hand, denitrosylases such as Trx or GSNOR, limit the influence of S-nitrosylation in certain areas and/or in certain protein targets.

mammals are mainly the NOS enzymes. Examples of subcellular compartmentalization of S-nitrosylation signaling have been described in neurons and cardiac tissue, where S-nitrosylated proteins with signaling functions co-localize with the different NOS isoforms. Similarly, in cells such as endothelial cells or T lymphocytes, eNOS-dependent S-nitrosylation is observed in proteins localized at the plasma membrane or on the Golgi, associated to regulated localization and activity of eNOS in these compartments.

A simple model of the short-range signaling exerted by S-nitrosylation in the NOS proximity is illustrated in Figure 1, where S-nitrosylation signaling is depicted in a limited spherical space around NOS. However, consideration of some of the mechanisms involved in S-nitrosothiol formation and breakage leads to a more complex picture, in which S-nitrosylation catalyzers (e.g., specific metal centers, membranes) or S-nitrosylation vectors (such as DNIC, transnitrosylases) could promote the expansion of the S-nitrosylation range of action, while the presence of denitrosylases could restrict it (Fig. 6).

Future studies will confirm, modify or even refute this hypothesis, but we consider that the subcellular localization of protein S-nitrosylation needs to be taken into account when the functional role of this modification is studied. This is important also in proteomic studies that attempt to identify the S-nitrosoproteome, even though it poses an additional challenge to current methodologies.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, and Tymianski M. Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science* 298: 846–850, 2002.
2. Aarts MM, and Tymianski M. Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. *Curr Mol Med* 4: 137–147, 2004.
3. Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schoneich C, and Cohen RA. S-glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med* 10: 1200–1207, 2004.
4. Ahern GP, Klyachko VA, and Jackson MB. cGMP and S-nitrosylation: Two routes for modulation of neuronal excitability by NO. *Trends Neurosci* 25: 510–517, 2002.
5. Anand P, and Stamler JS. Enzymatic mechanisms regulating protein S-nitrosylation: Implications in health and disease. *J Mol Med (Berl)* 90: 233–244, 2012.
6. Araújo IM, Ambrósio AF, Leal EC, Santos PF, Carvalho AP, and Carvalho CM. Neuronal nitric oxide synthase proteolysis limits the involvement of nitric oxide in kainate-induced neurotoxicity in hippocampal neurons. *J Neurochem* 85: 791–800, 2003.
7. Araújo IM, and Carvalho CM. Role of nitric oxide and calpain activation in neuronal death and survival. *Curr Drug Targets CNS Neurol Disord* 4: 319–324, 2005.
8. Arner ES, and Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267: 6102–6109, 2000.
9. Arstall MA, Sawyer DB, Fukazawa R, and Kelly RA. Cytokine-mediated apoptosis in cardiac myocytes: The role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ Res* 85: 829–840, 1999.
10. Arundine M, and Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* 34: 325–337, 2003.
11. Awad HH, and Stanbury DM. Autoxidation of NO in aqueous solution. *Intl J Chem Kinetics* 25: 375–381, 1993.
12. Balligand JL, Ungureanu-Longrois D, Simmons WW, Kobzik L, Lowenstein CJ, Lamas S, Kelly RA, Smith TW, and Michel T. Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1 beta and IFN-gamma. *Am J Physiol* 268: H1293–1303, 1995.
13. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, and Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* 416: 337–339, 2002.
14. Bateman RL, Rauh D, Tavshanjian B, and Shokat KM. Human carbonyl reductase 1 is an S-nitrosoglutathione reductase. *J Biol Chem* 283: 35756–35762, 2008.
15. Batista WL, Ogata FT, Curcio MF, Miguel RB, Arai RJ, Matsuo AL, Moraes MS, Stern A, and Monteiro HP. S-nitrosoglutathione and endothelial nitric oxide synthase-derived nitric oxide regulate compartmentalized Ras S-nitrosylation and stimulate cell proliferation. *Antioxid Redox Signal* 18: 221–238, 2013.
16. Benhar M, Forrester MT, Hess DT, and Stamler JS. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* 320: 1050–1054, 2008.
17. Benhar M, Forrester MT, and Stamler JS. Protein denitrosylation: Enzymatic mechanisms and cellular functions. *Nat Rev Mol Cell Biol* 10: 721–732, 2009.
18. Benhar M, Thompson JW, Moseley MA, and Stamler JS. Identification of S-nitrosylated targets of thioredoxin using a quantitative proteomic approach. *Biochemistry* 49: 6963–6969, 2010.
19. Berthiaume LG. Insider information: How palmitoylation of Ras makes it a signaling double agent. *Sci STKE* 2002: pe41, 2002.
20. Bosworth CA, Toledo JC, Jr., Zmijewski JW, Li Q, and Lancaster JR, Jr. Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. *Proc Natl Acad Sci USA* 106: 4671–4676, 2009.
21. Bredt DS, and Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc Natl Acad Sci USA* 86: 9030–9033, 1989.

22. Brenman JE, and Bredt DS. Synaptic signaling by nitric oxide. *Curr Opin Neurobiol* 7: 374–378, 1997.
23. Broniowska KA, and Hogg N. The chemical biology of S-nitrosothiols. *Antioxid Redox Signal* 17: 969–980, 2012.
24. Calabrese V, Boyd-Kimball D, Scapagnini G, and Butterfield DA. Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: The role of vitagenes. *In Vivo* 18: 245–267, 2004.
25. Cao S, Yao J, McCabe TJ, Yao Q, Katusic ZS, Sessa WC, and Shah V. Direct interaction between endothelial nitric-oxide synthase and dynamin-2. Implications for nitric-oxide synthase function. *J Biol Chem* 276: 14249–14256, 2001.
26. Cary SP, Winger JA, Derbyshire ER, and Marletta MA. Nitric oxide signaling: No longer simply on or off. *Trends Biochem Sci* 31: 231–239, 2006.
27. Chabrier PE, Demerle-Pallardy C, and Auguet M. Nitric oxide synthases: Targets for therapeutic strategies in neurological diseases. *Cell Mol Life Sci* 55: 1029–1035, 1999.
28. Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z, and Lipton SA. S-Nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. *Science* 324: 102–105, 2009.
29. Choi YB, and Lipton SA. Redox modulation of the NMDA receptor. *Cell Mol Life Sci* 57: 1535–1541, 2000.
30. Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, and Lipton SA. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat Neurosci* 3: 15–21, 2000.
31. Christopherson KS, Hillier BJ, Lim WA, and Bredt DS. PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J Biol Chem* 274: 27467–27473, 1999.
32. Church JE, and Fulton D. Differences in eNOS activity because of subcellular localization are dictated by phosphorylation state rather than the local calcium environment. *J Biol Chem* 281: 1477–1488, 2006.
33. D'Mello R, Marchand F, Pezet S, McMahon SB, and Dickenson AH. Perturbing PSD-95 interactions with NR2B-subtype receptors attenuates spinal nociceptive plasticity and neuropathic pain. *Mol Ther* 19: 1780–1792, 2011.
34. Dawson VL, Dawson TM, London ED, Bredt DS, and Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 88: 6368–6371, 1991.
35. Derakhshan B, Hao G, and Gross SS. Balancing reactivity against selectivity: The evolution of protein S-nitrosylation as an effector of cell signaling by nitric oxide. *Cardiovasc Res* 75: 210–219, 2007.
36. Dudzinski DM, and Michel T. Life history of eNOS: Partners and pathways. *Cardiovasc Res* 75: 247–260, 2007.
37. Erwin PA, Lin AJ, Golan DE, and Michel T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric oxide synthase in vascular endothelial cells. *J Biol Chem* 280: 19888–19894, 2005.
38. Erwin PA, Mitchell DA, Sartoretto J, Marletta MA, and Michel T. Subcellular targeting and differential S-nitrosylation of endothelial nitric oxide synthase. *J Biol Chem* 281: 151–157, 2006.
39. Ferrante RJ, Kowall NW, Beal MF, Richardson EP, Jr., Bird ED, and Martin JB. Selective sparing of a class of striatal neurons in Huntington's disease. *Science* 230: 561–563, 1985.
40. Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, and Sessa WC. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* 90: 866–873, 2002.
41. Forder JP, and Tymianski M. Postsynaptic mechanisms of excitotoxicity: Involvement of postsynaptic density proteins, radicals, and oxidant molecules. *Neuroscience* 158: 293–300, 2009.
42. Forman HJ, Fukuto JM, and Torres M. Redox signaling: Thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol* 287: C246–256, 2004.
43. Forrester MT, Seth D, Hausladen A, Eyler CE, Foster MW, Matsumoto A, Benhar M, Marshall HE, and Stamler JS. Thioredoxin-interacting protein (Txnip) is a feedback regulator of S-nitrosylation. *J Biol Chem* 284: 36160–36166, 2009.
44. Foster MW, Hess DT, and Stamler JS. Protein S-nitrosylation in health and disease: A current perspective. *Trends Mol Med* 15: 391–404, 2009.
45. Fulton D, Babbitt R, Zoellner S, Fontana J, Acevedo L, McCabe TJ, Iwakiri Y, and Sessa WC. Targeting of endothelial nitric-oxide synthase to the cytoplasmic face of the Golgi complex or plasma membrane regulates Akt- versus calcium-dependent mechanisms for nitric oxide release. *J Biol Chem* 279: 30349–30357, 2004.
46. Fulton D, Gratton JP, and Sessa WC. Post-translational control of endothelial nitric oxide synthase: Why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther* 299: 818–824, 2001.
47. Garthwaite J, Charles SL, and Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336: 385–388, 1988.
48. Goldstein S, and Czapski G. Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants. The nature of the oxidizing intermediates. *J Am Chem Soc* 117: 12078–12084, 1995.
49. Gonzalez DR, Beigi F, Treuer AV, and Hare JM. Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes. *Proc Natl Acad Sci USA* 104: 20612–20617, 2007.
50. Gow AJ, and Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391: 169–173, 1998.
51. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, and Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem* 275: 22268–22272, 2000.
52. Gryglewski RJ, Palmer RM, and Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 320: 454–456, 1986.
53. Guikema B, Lu Q, and Jourd'heuil D. Chemical considerations and biological selectivity of protein nitrosation: Implications for NO-mediated signal transduction. *Antioxid Redox Signal* 7: 593–606, 2005.
54. Haendeler J, Hoffmann J, Tischler V, Berk BC, Zeiher AM, and Dimmeler S. Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nat Cell Biol* 4: 743–749, 2002.
55. Hammond J, and Balligand JL. Nitric oxide synthase and cyclic GMP signaling in cardiac myocytes: From

contractility to remodeling. *J Mol Cell Cardiol* 52: 330–340, 2012.
56. Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, Takahashi M, Cheah JH, Tankou SK, Hester LD, Ferris CD, Hayward SD, Snyder SH, and Sawa A. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat Cell Biol* 7: 665–674, 2005.
57. Hare JM, Lofthouse RA, Juang GJ, Colman L, Ricker KM, Kim B, Senzaki H, Cao S, Tunin RS, and Kass DA. Contribution of caveolin protein abundance to augmented nitric oxide signaling in conscious dogs with pacing-induced heart failure. *Circ Res* 86: 1085–1092, 2000.
58. Harris LK, McCormick J, Cartwright JE, Whitley GS, and Dash PR. S-Nitrosylation of proteins at the leading edge of migrating trophoblasts by inducible nitric oxide synthase promotes trophoblast invasion. *Exp Cell Res* 314: 1765–1776, 2008.
59. Hashemy SI, and Holmgren A. Regulation of the catalytic activity and structure of human thioredoxin 1 via oxidation and S-nitrosylation of cysteine residues. *J Biol Chem* 283: 21890–21898, 2008.
60. Hernansanz-Agustín P, Izquierdo-Álvarez A, García-Ortiz A, Ibiza S, Serrador JM, and Martínez-Ruiz A. Nitrosothiols in the immune system: Signaling and protection. *Antioxid Redox Signal* 18: 288–308, 2013.
61. Hess DT, Matsumoto A, Kim S-O, Marshall HE, and Stamler JS. Protein S-nitrosylation: Purview and parameters. *Nat Rev Mol Cell Biol* 6: 150–166, 2005.
62. Hickok JR, Sahni S, Shen H, Arvind A, Antoniou C, Fung LW, and Thomas DD. Dinitrosyliron complexes are the most abundant nitric oxide-derived cellular adduct: Biological parameters of assembly and disappearance. *Free Radic Biol Med* 51: 1558–1566, 2011.
63. Ho GP, Selvakumar B, Mukai J, Hester LD, Wang Y, Gogos JA, and Snyder SH. S-Nitrosylation and S-palmitoylation reciprocally regulate synaptic targeting of PSD-95. *Neuron* 71: 131–141, 2011.
64. Hou Y, Guo Z, Li J, and Wang PG. Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. *Biochem Biophys Res Commun* 228: 88–93, 1996.
65. Huang Y, Man HY, Sekine-Aizawa Y, Han Y, Juluri K, Luo H, Cheah J, Lowenstein C, Huganir RL, and Snyder SH. S-nitrosylation of N-ethylmaleimide sensitive factor mediates surface expression of AMPA receptors. *Neuron* 46: 533–540, 2005.
66. Hyman BT, Marzloff K, Wenniger JJ, Dawson TM, Bredt DS, and Snyder SH. Relative sparing of nitric oxide synthase-containing neurons in the hippocampal formation in Alzheimer's disease. *Ann Neurol* 32: 818–820, 1992.
67. Ibiza S, Pérez-Rodríguez A, Ortega Á, Martínez-Ruiz A, Barreiro O, García-Domínguez CA, Víctor VM, Esplugues JV, Rojas JM, Sánchez-Madrid F, and Serrador JM. Endothelial nitric oxide synthase regulates N-Ras activation on the Golgi complex of antigen-stimulated T cells. *Proc Natl Acad Sci USA* 105: 10507–10512, 2008.
68. Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 84: 9265–9269, 1987.
69. Ischiropoulos H. Protein tyrosine nitration—An update. *Arch Biochem Biophys* 484: 117–121, 2009.
70. Iwakiri Y. S-Nitrosylation of proteins: A new insight into endothelial cell function regulated by eNOS-derived NO. *Nitric Oxide* 25: 95–101, 2011.
71. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, and Sessa WC. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. *Proc Natl Acad Sci USA* 103: 19777–19782, 2006.
72. Jagnandan D, Sessa WC, and Fulton D. Intracellular location regulates calcium-calmodulin-dependent activation of organelle-restricted eNOS. *Am J Physiol Cell Physiol* 289: C1024–1033, 2005.
73. Janssen-Heininger YM, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T, Stamler JS, Rhee SG, and van der Vliet A. Redox-based regulation of signal transduction: Principles, pitfalls, and promises. *Free Radic Biol Med* 45: 1–17, 2008.
74. Jarabek BR, Yasuda RP, and Wolfe BB. Regulation of proteins affecting NMDA receptor-induced excitotoxicity in a Huntington's mouse model. *Brain* 127: 505–516, 2004.
75. Jia L, Bonaventura C, Bonaventura J, and Stamler JS. S-nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* 380: 221–226, 1996.
76. Jourd'heuil D, Jourd'heuil FL, and Feelisch M. Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism. *J Biol Chem* 278: 15720–15726, 2003.
77. Jourd'heuil D, Laroux FS, Miles AM, Wink DA, and Grisham MB. Effect of superoxide dismutase on the stability of S-nitrosothiols. *Arch Biochem Biophys* 361: 323–330, 1999.
78. Keynes RG, and Garthwaite J. Nitric oxide and its role in ischaemic brain injury. *Curr Mol Med* 4: 179–191, 2004.
79. Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnelle DR, Stamler JS, and Lipton SA. Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO. *Neuron* 24: 461–469, 1999.
80. Klatt P, and Lamas S. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944., 2000.
81. Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM, Law L, Hester LD, and Snyder SH. GAPDH mediates nitrosylation of nuclear proteins. *Nat Cell Biol* 12: 1094–1100, 2010.
82. Lancaster JR, Jr. Nitroxidative, nitrosative, and nitrative stress: Kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chem Res Toxicol* 19: 1160–1174, 2006.
83. Lancaster JR Jr, and Gaston B. NO and nitrosothiols: Spatial confinement and free diffusion. *Am J Physiol Lung Cell Mol Physiol* 287: L465–466, 2004.
84. Lau A, and Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch* 460: 525–542, 2010.
85. Lei SZ, Pan ZH, Aggarwal SK, Chen HS, Hartman J, Sucher NJ, and Lipton SA. Effect of nitric oxide production on the redox modulatory site of the NMDA receptor–channel complex. *Neuron* 8: 1087–1099, 1992.
86. Lillig CH, and Holmgren A. Thioredoxin and related molecules—From biology to health and disease. *Antioxid Redox Signal* 9: 25–47, 2007.
87. Lim CH, Dedon PC, and Deen WM. Kinetic analysis of intracellular concentrations of reactive nitrogen species. *Chem Res Toxicol* 21: 2134–2147, 2008.
88. Lima B, Forrester MT, Hess DT, and Stamler JS. S-Nitrosylation in cardiovascular signaling. *Circ Res* 106: 633–646, 2010.

89. Lima B, Lam GKW, Xie L, Diesen DL, Villamizar N, Nienaber J, Messina E, Bowles D, Kontos CD, Hare JM, Stamler JS, and Rockman HA. Endogenous S-nitrosothiols protect against myocardial injury. *Proc Natl Acad Sci USA* 106: 6297–6302, 2009.
90. Lindermayr C, and Durner J. S-Nitrosylation in plants: Pattern and function. *J Proteomics* 73: 1–9, 2009.
91. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, and Stamler JS. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364: 626–632, 1993.
92. Lipton SA, Rayudu PV, Choi YB, Sucher NJ, and Chen HS. Redox modulation of the NMDA receptor by NO-related species. *Prog Brain Res* 118: 73–82, 1998.
93. Lipton SA, and Stamler JS. Actions of redox-related congeners of nitric oxide at the NMDA receptor. *Neuropharmacology* 33: 1229–1233, 1994.
94. Liu J, García-Cardeña G, and Sessa WC. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: Implications for caveolae localization. *Biochemistry* 35: 13277–13281, 1996.
95. Liu L, Hausladen A, Zeng M, Que L, Heitman J, and Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 410: 490–494, 2001.
96. Liu L, Yan Y, Zeng M, Zhang J, Hanes MA, Ahearn G, McMahon TJ, Dickfeld T, Marshall HE, Que LG, and Stamler JS. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 116: 617–628, 2004.
97. Liu X, Miller MJ, Joshi MS, Thomas DD, and Lancaster JR, Jr. Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci USA* 95: 2175–2179, 1998.
98. Lu W, Man H, Ju W, Trimble WS, MacDonald JF, and Wang YT. Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* 29: 243–254, 2001.
99. Malik M, Jividen K, Padmakumar VC, Cataisson C, Li L, Lee J, Howard OM, and Yuspa SH. Inducible NOS-induced chloride intracellular channel 4 (CLIC4) nuclear translocation regulates macrophage deactivation. *Proc Natl Acad Sci USA* 109: 6130–6135, 2012.
100. Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, and Stamler JS. Fas-induced caspase denitrosylation. *Science* 284: 651–654, 1999.
101. Marino SM, and Gladyshev VN. Structural analysis of cysteine S-nitrosylation: A modified acid-based motif and the emerging role of trans-nitrosylation. *J Mol Biol* 395: 844–859, 2010.
102. Maron BA, Tang SS, and Loscalzo J. S-nitrosothiols and the S-nitrosoproteome of the cardiovascular system. *Antioxid Redox Signal* 18: 270–287, 2013.
103. Martínez-Ruiz A, Cadenas S, and Lamas S. Nitric oxide signaling: Classical, less classical, and nonclassical mechanisms. *Free Radic Biol Med* 51: 17–29, 2011.
104. Martínez-Ruiz A, and Lamas S. S-Nitrosylation: A potential new paradigm in signal transduction. *Cardiovasc Res* 62: 43–52, 2004.
105. Martínez-Ruiz A, and Lamas S. Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences. *Cardiovasc Res* 75: 220–228, 2007.
106. Martínez-Ruiz A, and Lamas S. Two decades of new concepts in nitric oxide signaling: From the discovery of a gas messenger to the mediation of nonenzymatic posttranslational modifications. *IUBMB Life* 61: 91–98, 2009.
107. Martínez-Ruiz A, Villanueva L, de Orduña CG, López-Ferrer D, Higueras MÁ, Tarín C, Rodríguez-Crespo I, Vázquez J, and Lamas S. S-Nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. *Proc Natl Acad Sci USA* 102: 8525–8530, 2005.
108. Massion PB, Feron O, Dessy C, and Balligand J-L. Nitric oxide and cardiac function: Ten years after, and continuing. *Circ Res* 93: 388–398, 2003.
109. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, and Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 115: 139–150, 2003.
110. McCormick ME, Goel R, Fulton D, Oess S, Newman D, and Tzima E. Platelet-endothelial cell adhesion molecule-1 regulates endothelial NO synthase activity and localization through signal transducers and activators of transcription 3-dependent NOSTRIN expression. *Arterioscler Thromb Vasc Biol* 31: 643–649, 2011.
111. Mieyal JJ, Gallogly MM, Qanungo S, Sabens EA, and Shelton MD. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxid Redox Signal* 10: 1941–1988, 2008.
112. Mitchell DA, and Marletta MA. Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. *Nat Chem Biol* 1: 154–158, 2005.
113. Möller MN, Li Q, Lancaster JR Jr, and Denicola A. Acceleration of nitric oxide autoxidation and nitrosation by membranes. *IUBMB Life* 59: 243–248, 2007.
114. Möller MN, Li Q, Vitturi DA, Robinson JM, Lancaster JR, Jr, and Denicola A. Membrane "lens" effect: Focusing the formation of reactive nitrogen oxides from the *NO/O2 reaction. *Chem Res Toxicol* 20: 709–714, 2007.
115. Moreno-López B, and González-Forero D. Nitric oxide and synaptic dynamics in the adult brain: Physiopathological aspects. *Rev Neurosci* 17: 309–357, 2006.
116. Morrell CN, Matsushita K, Chiles K, Scharpf RB, Yamakuchi M, Mason RJ, Bergmeier W, Mankowski JL, Baldwin WM, 3rd, Faraday N, and Lowenstein CJ. Regulation of platelet granule exocytosis by S-nitrosylation. *Proc Natl Acad Sci USA* 102: 3782–3787, 2005.
117. Mukhopadhyay S, Lee J, and Sehgal PB. Depletion of the ATPase NSF from Golgi membranes with hypo-S-nitrosylation of vasorelevant proteins in endothelial cells exposed to monocrotaline pyrrole. *Am J Physiol Heart Circ Physiol* 295: H1943–1955, 2008.
118. Mukhopadhyay S, Xu F, and Sehgal PB. Aberrant cytoplasmic sequestration of eNOS in endothelial cells after monocrotaline, hypoxia, and senescence: Live-cell caveolar and cytoplasmic NO imaging. *Am J Physiol Heart Circ Physiol* 292: H1373–1389, 2007.
119. Nakamura T, and Lipton SA. Emerging role of protein–protein transnitrosylation in cell signaling pathways. *Antioxid Redox Signal* 18: 239–249, 2013.
120. Nakamura T, Wang L, Wong CC, Scott FL, Eckelman BP, Han X, Tzitzilonis C, Meng F, Gu Z, Holland EA, Clemente AT, Okamoto S, Salvesen GS, Riek R, Yates JR, 3rd, and Lipton SA. Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Mol Cell* 39: 184–195, 2010.

121. Nott A, Watson PM, Robinson JD, Crepaldi L, and Riccio A. S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature* 455: 411–415, 2008.
122. Oess S, Icking A, Fulton D, Govers R, and Müller-Esterl W. Subcellular targeting and trafficking of nitric oxide synthases. *Biochem J* 396: 401–409, 2006.
123. Ozawa K, Whalen EJ, Nelson CD, Mu Y, Hess DT, Lefkowitz RJ, and Stamler JS. S-Nitrosylation of beta-arrestin regulates beta-adrenergic receptor trafficking. *Mol Cell* 31: 395–405, 2008.
124. Pacher P, Beckman JS, and Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87: 315–424, 2007.
125. Paige JS, Xu G, Stancevic B, and Jaffrey SR. Nitrosothiol reactivity profiling identifies S-nitrosylated proteins with unexpected stability. *Chem Biol* 15: 1307–1316, 2008.
126. Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsey MZ, Forbes MS, Macdonald T, and Gaston B. S-nitrosothiols signal hypoxia-mimetic vascular pathology. *J Clin Invest* 117: 2592–2601, 2007.
127. Palmer RM, Ashton DS, and Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664–666, 1988.
128. Pawloski JR, Hess DT, and Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature* 409: 622–626, 2001.
129. Peng HM, Morishima Y, Pratt WB, and Osawa Y. Modulation of heme/substrate binding cleft of neuronal nitric-oxide synthase (nNOS) regulates binding of Hsp90 and Hsp70 proteins and nNOS ubiquitination. *J Biol Chem* 287: 1556–1565, 2012.
130. Pi X, Wu Y, Ferguson JE, 3rd, Portbury AL, and Patterson C. SDF-1alpha stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration. *Proc Natl Acad Sci USA* 106: 5675–5680, 2009.
131. Qian J, Zhang Q, Church JE, Stepp DW, Rudic RD, and Fulton DJ. Role of local production of endothelium-derived nitric oxide on cGMP signaling and S-nitrosylation. *Am J Physiol Heart Circ Physiol* 298: H112–118, 2010.
132. Qu J, Nakamura T, Cao G, Holland EA, McKercher SR, and Lipton SA. S-Nitrosylation activates Cdk5 and contributes to synaptic spine loss induced by beta-amyloid peptide. *Proc Natl Acad Sci USA* 108: 14330–14335, 2011.
133. Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci USA* 101: 4003–4008, 2004.
134. Radi R, Beckman JS, Bush KM, and Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266: 4244–4250, 1991.
135. Reiter E, and Lefkowitz RJ. GRKs and beta-arrestins: Roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab* 17: 159–165, 2006.
136. Sangwung P, Greco TM, Wang Y, Ischiropoulos H, Sessa WC, and Iwakiri Y. Proteomic identification of S-nitrosylated Golgi proteins: New insights into endothelial cell regulation by eNOS-derived NO. *PLoS One* 7: e31564, 2012.
137. Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, and Tymianski M. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284: 1845–1848, 1999.
138. Schulman IH, and Hare JM. Regulation of cardiovascular cellular processes by S-nitrosylation. *Biochim Biophys Acta* 1820: 752–762, 2012.
139. Seddon M, Shah AM, and Casadei B. Cardiomyocytes as effectors of nitric oxide signalling. *Cardiovasc Res* 75: 315–326, 2007.
140. Selvakumar B, Huganir RL, and Snyder SH. S-Nitrosylation of stargazin regulates surface expression of AMPA-glutamate neurotransmitter receptors. *Proc Natl Acad Sci USA* 106: 16440–16445, 2009.
141. Seth D, and Stamler JS. The SNO-proteome: Causation and classifications. *Curr Opin Chem Biol* 15: 129–136, 2011.
142. Singel DJ, and Stamler JS. Chemical physiology of blood flow regulation by red blood cells: The role of nitric oxide and S-nitrosohemoglobin. *Annu Rev Physiol* 67: 99–145, 2005.
143. Sliskovic I, Raturi A, and Mutus B. Characterization of the S-denitrosation activity of protein disulfide isomerase. *J Biol Chem* 280: 8733–8741, 2005.
144. Sossa KG, Beattie JB, and Carroll RC. AMPAR exocytosis through NO modulation of PICK1. *Neuropharmacology* 53: 92–100, 2007.
145. Souza JM, Peluffo G, and Radi R. Protein tyrosine nitration—Functional alteration or just a biomarker? *Free Rad Biol Med* 45: 357–366, 2008.
146. Sowa G, Liu J, Papapetropoulos A, Rex-Haffner M, Hughes TE, and Sessa WC. Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J Biol Chem* 274: 22524–22531, 1999.
147. Stamler JS, and Toone EJ. The decomposition of thionitrites. *Curr Opin Chem Biol* 6: 779–785, 2002.
148. Takahashi H, Shin Y, Cho S-J, Zago WM, Nakamura T, Gu Z, Ma Y, Furukawa H, Liddington R, Zhang D, Tong G, Chen H-SV, and Lipton SA. Hypoxia enhances S-nitrosylation-mediated NMDA receptor inhibition via a thiol oxygen sensor motif. *Neuron* 53: 53–64, 2007.
149. Takahashi S, and Mendelsohn ME. Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and Akt: calcium-independent eNOS activation involves formation of an HSP90-Akt-CaM-bound eNOS complex. *J Biol Chem* 278: 30821–30827, 2003.
150. Tamargo J, Caballero R, Gómez R, and Delpón E. Cardiac electrophysiological effects of nitric oxide. *Cardiovasc Res* 87: 593–600, 2010.
151. Tegeder I, Scheving R, Wittig I, and Geisslinger G. SNO-ing at the nociceptive synapse? *Pharmacol Rev* 63: 366–89, 2011.
152. Tello D, Tarín C, Ahicart P, Bretón-Romero R, Lamas S, and Martínez-Ruiz A. A "fluorescence switch" technique increases the sensitivity of proteomic detection and identification of S-nitrosylated proteins. *Proteomics* 9: 5359–5370, 2009.
153. Thibeault S, Rautureau Y, Oubaha M, Faubert D, Wilkes BC, Delisle C, and Gratton JP. S-Nitrosylation of beta-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol Cell* 39: 468–476, 2010.
154. Trujillo M, Alvarez MN, Peluffo G, Freeman BA, and Radi R. Xanthine oxidase-mediated decomposition of S-nitrosothiols. *J Biol Chem* 273: 7828–7834, 1998.
155. Tsang AH, Lee YI, Ko HS, Savitt JM, Pletnikova O, Troncoso JC, Dawson VL, Dawson TM, and Chung KK. S-Nitrosylation of XIAP compromises neuronal survival in Parkinson's disease. *Proc Natl Acad Sci USA* 106: 4900–4905, 2009.
156. Umar S, and van der Laarse A. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic, and failing heart. *Mol Cell Biochem* 333: 191–201, 2010.

157. Viner RI, Williams TD, and Schöneich C. Peroxynitrite modification of protein thiols: Oxidation, nitrosylation, and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* 38: 12408–12415, 1999.
158. Wang G, Moniri NH, Ozawa K, Stamler JS, and Daaka Y. Nitric oxide regulates endocytosis by S-nitrosylation of dynamin. *Proc Natl Acad Sci USA* 103: 1295–1300, 2006.
159. Wang X, Su B, Zheng L, Perry G, Smith MA, and Zhu X. The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer's disease. *J Neurochem* 109 Suppl 1: 153–159, 2009.
160. Wang Y, Yun BW, Kwon E, Hong JK, Yoon J, and Loake GJ. S-Nitrosylation: An emerging redox-based post-translational modification in plants. *J Exp Bot* 57: 1777–1784, 2006.
161. Wang Z, Humphrey C, Frilot N, Wang G, Nie Z, Moniri NH, and Daaka Y. Dynamin2- and endothelial nitric oxide synthase-regulated invasion of bladder epithelial cells by uropathogenic *Escherichia coli*. *J Cell Biol* 192: 101–110, 2011.
162. Wei W, Li B, Hanes MA, Kakar S, Chen X, and Liu L. S-nitrosylation from GSNOR deficiency impairs DNA repair and promotes hepatocarcinogenesis. *Sci Transl Med* 2: 19ra13, 2010.
163. West MB, Hill BG, Xuan YT, and Bhatnagar A. Protein glutathiolation by nitric oxide: an intracellular mechanism regulating redox protein modification. *FASEB J* 20: 1715–1717, 2006.
164. Whalen EJ, Foster MW, Matsumoto A, Ozawa K, Violin JD, Que LG, Nelson CD, Benhar M, Keys JR, Rockman HA, Koch WJ, Daaka Y, Lefkowitz RJ, and Stamler JS. Regulation of [beta]-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. *Cell* 129: 511–522, 2007.
165. Wink DA, Darbyshire JF, Nims RW, Saavedra JE, and Ford PC. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* 6: 23–27, 1993.
166. Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, Parrott AM, Baykal AT, Sadoshima J, and Li H. Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* 9: 2262–2275, 2010.
167. Wu C, Parrott AM, Fu C, Liu T, Marino SM, Gladyshev VN, Jain MR, Baykal AT, Li Q, Oka S, Sadoshima J, Beuve A, Simmons WJ, and Li H. Thioredoxin 1-mediated post-translational modifications: Reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. *Antioxid Redox Signal* 15: 2565–2604, 2011.
168. Wu C, Parrott AM, Liu T, Jain MR, Yang Y, Sadoshima J, and Li H. Distinction of thioredoxin transnitrosylation and denitrosylation target proteins by the ICAT quantitative approach. *J Proteomics* 74: 2498–2509, 2011.
169. Yoshida M, and Xia Y. Heat shock protein 90 as an endogenous protein enhancer of inducible nitric-oxide synthase. *J Biol Chem* 278: 36953–36958, 2003.
170. Zhang Q, Church JE, Jagnandan D, Catravas JD, Sessa WC, and Fulton D. Functional relevance of Golgi- and plasma membrane-localized endothelial NO synthase in reconstituted endothelial cells. *Arterioscler Thromb Vasc Biol* 26: 1015–1021, 2006.
171. Zucker B, Luthi-Carter R, Kama JA, Dunah AW, Stern EA, Fox JH, Standaert DG, Young AB, and Augood SJ. Transcriptional dysregulation in striatal projection- and interneurons in a mouse model of Huntington's disease: Neuronal selectivity and potential neuroprotective role of HAP1. *Hum Mol Genet* 14: 179–189, 2005.

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Abbreviations Used

AD = Alzheimer disease
β_2-AR = β_2-adrenergic receptor
eNOS = endothelial nitric oxide synthase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GPCR = G protein-coupled receptors
GRK = GPCR kinase
GSH = reduced glutathione
GSNO = S-nitrosoglutathione
GSNOR = S-nitrosoglutathione reductase
Hb = hemoglobin
HD = Huntington disease
iNOS = inducible nitric oxide synthase
LMM = low molecular mass
LPS = lipopolysaccharide
LTCC = L-type Ca^{2+} channel
nNOS = neuronal nitric oxide synthase
NO = nitric oxide
NOS = nitric oxide synthase
$ONOO^-$ = peroxynitrite
PTM = post-translational modification
RNS = reactive nitrogen species
ROS = reactive oxygen species
RyR = ryanodine receptor
SERCA = sarcoplasmic/endoplasmic reticulum Ca^{2+}-activated ATPase
sGC = soluble guanylate cyclase
SNO-protein = S-nitrosylated protein
TR = thioredoxin reductase
Trx = thioredoxin